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Molecular epidemiology and genetic characterization of *Clostridium perfringens* infections in lambs

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ABSTRACT

Clostridium perfringens is gram positive bacterium, wide spread in environment causing various diseases in animals and human. The current study was conducted to indentify the genetic identity of *C. perfringens* isolates from lambs from Egypt. Using specific primers amplifying genes associated to the toxins produced by *C. perfringens*, multiplex PCR was used to confirm *C. perfringens* in 87 out of 140 samples were collected from diseased and suspected lambs. The isolates were classified as type A in 49.4%, type B in 31.1% and type D in 19.5% of isolates. The phylogenetic analysis for the partial sequences of *C. perfringens* strains based on *plc* gene, *cpb* gene and *etx* gene obtained in the present study showed high degree of similarity with other sequences of *C. perfringens* strains in GenBank, isolating from sheep from Egypt and other countries. According to the findings, lambs with enterotoxaemia more frequently have *C. perfringens* type A and an efficient hygienic control program is necessary to reduce the infection spreading among susceptible animals.

1. Introduction

Clostridium perfringens (*C. perfringens*) is an anaerobic, gram-positive bacterium that causes a variety of diseases in animals and is typically seen in animals' gastrointestinal tracts [1,2]. *C. perfringens* is one of the main pathogens responsible for intestinal infections as well as histotoxic diseases in animals, having a low incidence rate (2–8%) but a very high case fatality rate (100%) [3,4]. The virulence of *C. perfringens* is dependent on the production of at least 20 different toxins and extracellular enzyme [5].

The pathogenicity has been primarily attributed to the release of highly destructive exotoxins, which serve as the basis for dividing the species into serotypes [6]. *C. perfringens* has been classified into five toxigenic types (A, B, C, D, and E) based on the expression of four major toxins, namely alpha (α), beta (β), epsilon (ε), and iota (ι) [7,8]. Each toxin type is connected to particular intestinal diseases in different animal species [9]. All toxinotypes generate the α-toxin, while type B and C strains create the β-toxin, type B and D strains produce the ε-toxin, and type E strains produce the ι–toxin [10,11]. The *cpa*, *cpb*, *etx*, and *iap* genes are encode the α-, β-, ε-, and ι-toxins, respectively [9,12]. The gene encoding *cpa* toxin is produced by all types, *cpb* is produced by types B and C, *etx* is produced by types B and D, while *iap* is produced by type E [13].

The *cpa* is the most studied *C. perfringens* toxin and it is an important immunogenic antigen involved in the pathogenesis of enterotoxaemia, as well as in the induction of necrotic lesions in the calf intestinal loop mode [14]. The *cpb* is produced by type B and C of *C. perfringens* and is considered to be responsible for fatal hemorrhagic dysentery in sheep (type B) and the fatal intestinal necrosis seen in type C infections in several animal species and humans [15]. The *etx* toxin is produced as a relatively inactive protoxin of 33 kDa that needs proteolytic activation from intestinal proteases to obtain its full functionality, produced by type B and D and is a potent pore-forming protein responsible for the neurologic signs [16].

The PCR genotyping provides a useful alternative to in vivo toxin neutralization tests for typing *C. perfringens* isolates. Genotypes can, in many cases, provide the final piece of information needed to establish a diagnosis [15]. Different PCR protocols were employed to genotype *C. perfringens* isolates with respect to the genes cpa, cpb, etx, iap, cpe, NetB, and cpb2, which encode the *α-, β-, ε-, ι*-toxins, *CPE, NetB*, and *β2*-toxins, respectively [17]. The multiplex PCR allows simultaneous detection and amplification of more than one gene target in one reaction

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and provides higher sensitivity over conventional PCR [17,18].

The present study aimed to identify *C. perfringens* isolating from lambs using multiplex PCR and determine the sequence variation in *α*-, *ε*-, and *β*-toxins producing isolates.

2. Material and methods

2.1. Animals and sampling

The study was carried out during 2021 for molecular investigation of *C. perfringens* isolates in lambs. A total of 140 fecal swabs from lambs aged one month were randomly taken using a simple random technique; 40 of them displayed severe diarrhea, while the other lambs appeared normal. The samples were collected from males and females during different seasons of year. The samples were placed in sterile separate polyethylene bags, labeled, and transported to the laboratory in icebox.

2.2. Extraction of DNA and PCR analysis

The DNA was extracted from the prepared fecal samples according to the manufacturer's instructions using the DNA stool Kit (Bioneer, South Korea). All of the DNA extracts were stored at - 20 °C until use. Amplification of target genes for the detection of *C. perfringens* (*16S rRNA*) was performed using the specific primers described in Table 1. All PCR amplifications were performed in 25-μL volumes containing 3 μL of DNA template, 0.5-mM concentrations of deoxynucleoside triphosphates, 2.5 μL of 10 × PCR buffer (gene fanavaran), 0.75 mM MgCl2, 0.3 μM concentrations of each forward and reverse primer, 0.2 U of Taq DNA polymerase (Gene Fanavaran, Iran) under the following conditions: initial denaturation step at 95 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 1 min, annealing for 1 min, and extension at 72 °C for 1 min. After the last cycle, the mixture was incubated at 72 °C for 5 min. The amplification products were analyzed by electrophoresis on a 1.5% agarose gel.

The multiplex PCR was carried out using specific six pair of primers sets in Table 1 for each *C. perfringens* toxin to identify *C. perfringens* types.

The multiplex PCR assay was carried out in final volume of 25 μL, each reaction mixture contained 1X PCR Buffer (Fermentas, Lithuania), 250 μM dNTPs (Invitrogen.TM grand Island, NY), 4 mM MgCl2 (Fermentas, Lithuania), 1.25 U Taq DNA polymerase (Fermentas, Lithuania), 0.12 μM forward and reverse primers of alpha, beta, epsilon, iota and entero gene, 0.16 μM forward and reverse primer of beta2 gene, 2 μL of sample DNA (150–200 ng/μL). The PCR condition for each toxin was described in details in Table 2. The PCR products were separated visualized on 2% stained ethidium bromide agarose gel.

Table 1
Primers used for amplification of different genes of *C. perfringens*.

Toxin	Sequence	Amplified product	Reference
Alpha toxin	GTTGATAGCGCAGGACATGTTAAG CATGTAGTCATCTGTTCCAGCATC	402 bp	[26]
Beta toxin	ACTATACAGACAGATCATTCAACC TTAGGAGCAGTTAGAACTACAGAC	236 bp	
Epsilon toxin	ACTGCAACTACTACTCATACTGTG CTGGTGCCTTAATAGAAAGACTCC	541 bp	
Cpb2	GAA AGG TAA TGG AGA ATT ATC TTA ATG C GCA GAA TCA GGA TTT TGA CCA TAT ACC	573 bp	[46]
Cpe	ACATCTGCAGATAGCTTAGGAAAT CCAGTAGCTGTAATTGTTAAGTGT	247 bp	[47]
16S RrNA	AAAGATGGCATCATCATTCAAC TACCGTCATTATCTTCCCCAAA	279 bp	[48]

2.3. DNA sequencing and phylogenetic analysis

The PCR products of positive samples were purified using QIAquick PCR product extraction kit (Qiagen, Valencia, USA), followed by sequencing using the same primers of conventional PCR. The sequencing was performed using big dye Terminator V3.1 cycle sequencing kit (PerkinElmer, Foster city, USA) in Applied Biosystems 3130 genetic analyze (ABI, 3130, USA) according to instruction of manufacture.

The sequences of different genes were aligned with other published sequences in GenBank using the CLUSTAL W multiple sequence alignment program and Phylogenetic analyses were constructed using maximum likelihood, neighbor joining and maximum parsimony in MEGA6 [19].

3. Results

Of 140 samples obtained from lambs, 87 (62.14%) were detected as *C. perfringens* by PCR.

Molecular genotyping of 87 isolates showed 43 (49.4%) type A, 27 (31.1%) type B and 17 (19.5%) type D.

Regarding to seasonal effect on occurrence of *C. perfringens*, the percentage rate in examine showed significant disparity ($P < 0.01$) between different seasons. The highest occurrence rate was observed in winter, autumn (79.03%, 75% respectively) followed by summer season was 51.72%, and the lowest occurrence rate was in spring 34.38%, Table 3.

Concerning to sex effect of occurrence of *C. perfringens*, the highest occurrence rate was observed in males 69.35% compared to females 56.41%, Table 3.

In relation to age effect on occurrence of *C. perfringens*, the highest occurrence rate was at one weeks of age of examined lambs 70.21%, Table 3.

The genotypes of 87 *C. perfringens* isolates from lambs were determined using multiplex PCR as demonstrated in Fig. 1. Of these isolates, 43 (49.4%) was type A (had only *plc* gene), 27 (31.1%) were type B (had *cpb*, *plc etx* and genes), and 17 (19.5%) was type D (had *plc* and *etx* genes), Table 4. However, all isolates gave negative results for *cpb2* and *cpe* genes.

The phylogenetic tree was constructed based on partial sequences of *plc* gene for three *C. perfringens* sequences using the Neighbor-joining methods with bootstrap probability more than 50%. It showed clear clustering with Egyptian strains and other *C. perfringens* strains from GenBank. The three strains of *C. perfringens* based on *plc* gene (OM854781, OM854783 and OM854782) were clustered together with *C. perfringens* strains from sheep from Saudi Arabia MN646348, MN646363, MN646364, MN646365 and MN646362, Fig. 2.

Moreover, the phylogenetic analysis for partial sequences of *cpb* gene using the Neighbor-joining methods with bootstrap probability more than 30% was performed and the obtained ON081025 strain in this study was clustered with KP064404 from USA and MN683525 from Sudia Arabia, Fig. 3. In addition, the *etx* gene of ON081027 strain was closely related to KY938006 from India, while ON081026 strain was clustered with KY938004, KY938005 and KY938007KY938006 from India and with JX010451 from USA, Fig. 4.

4. Discussion

C. perfringens is a cause of enterotoxaemia, affecting sheep and goats causing great economic losses in these animals throughout the world due to high prevalence and fatality rates [15]. It's crucial to type *C. perfringens* strains since various bacterial species are associated with specific enteric diseases in animals [20]. The PCR is the best choice for typing and sub-typing. Various PCR protocols have been established for the typing of *C. perfringens* isolates [21]. In the current study, toxino-typing was performed using multiplex PCR for investigation of three toxin genes, *plc, cpb* and *etx* coded for (α, β, ε toxins, respectively)

Table 2
Thermal conditions of the different primers using in PCR assay.

Toxin	Primary denaturation	Secondary denaturation	Annealing	Extension	No. of cycles	Final extension
Alpha, Beta, Epsilon,	94 °C 5 min.	94 °C 1 min.	55 °C 1 min.	72 °C 1 min.	35	72 °C 10 min.
Cpb2	94 °C 5 min.	94 °C 30 s.	48 °C 40 s.	72 °C 45 s.	35	72 °C 10 min.
cpe	94 °C 5 min.	94 °C 30 s.	55 °C 45 s.	72 °C 45 s.	35	72 °C 10 min.
16S rRNA	94 °C 5 min.	94 °C 30 s.	53 °C 30 s.	72 °C 30 s.	35	72 °C 7 min.

Table 3
Results of examined lambs based on PCR assay.

Variable	No of examined lamb	No of positive	% of positive	95%CI	*P* value
Sex					
Male	62	43	69.35	57.03–79.42	0.681
Female	78	44	56.41	45.36–66.86	
Age					
One week	47	33	70.21	56.02–81.35	0.417
Two weeks	36	23	63.89	47.58–77.53	
Three weeks	41	22	53.66	38.75–67.95	
Four weeks	16	9	56.25	33.18–76.9	
Season					
Winter	62	49	79.03	67.36–87.31	<0.0001
Spring	32	11	34.38	20.41–51.69	
Summer	29	15	51.72	34.43–68.61	
Autumn	16	12	75	50.5–89.82	
Total	140	87	62.14	53.88–69.75	

The results is significant if *P* value < 0.05.

and typing of the detected *C. perfringens* isolates in lambs.

The overall occurrence of *C. perfringens* in examined lambs was 62.14%, which was higher than the rates of 56.16% and 59.62% previously reported in India by Nazki, Wani [22] and Kumar, Sreenivasulu [23] but lower than rate of 80.73% reported in Pakistan by Hussain, Muhammad [24]. This difference in prevalence between different countries may be contributed to sample size, diagnostic technique and species of examined animals [25].

Interestingly, the predominant *C. perfringens* toxovar was type A 449.4% followed by *C. perfringens* type B and D (31.1%, 19.5% respectively). According to our findings, a number of studies indicated that type A of *C. perfringens* is the most prevalent type worldwide [26], as reported in Pakistan (82.0%) [27] and Italy (93.0%) [28], while the other types were scarce.

These results differ from those of Nazki, Wani [22], who discovered that 70.84% of isolates were detected as toxin type D and 29.16% of isolates were toxin type A. In contrast to the current results, the prevalence of *C. perfringens* type A was found to be quite low in lambs suspected of having the disease, at only 5.13% [29]. These relative variations in *C. perfringens* type rates could be due to sampling techniques, isolation methodologies, the time of year when samples were taken, or other unknown variables.

The winter and autumn seasons in the present study had the highest prevalence rates (79.03% and 75%, respectively), the summer season coming in second at 51.72% and the lowest prevalence rate was in the spring, which was consistent with Omar [30], who found that *C. perfringens* prevalences were greater in the winter and autumn seasons (73%) and lower in the spring and summer seasons (27%). In contrast, Khan, Durrani [31] observed different results, finding that in lambs, the highest prevalence of *C. perfringens* type A (23.81%) was recorded during the spring, followed by winter (14.29%) and autumn (11.90%), while the lowest prevalence (4.76%) was documented during summer.

Table 4
Toxin types and genes of identified *C. perfringens* isolates.

C. perfringens toxin type	Toxin gene	No of isolates	% of isolates
A	*Plc*	43	49.4
B	*Cpb*, *plc* and *etx*	27	31.1
D	*Plc*, *etx*	17	19.5
Total		87	100%

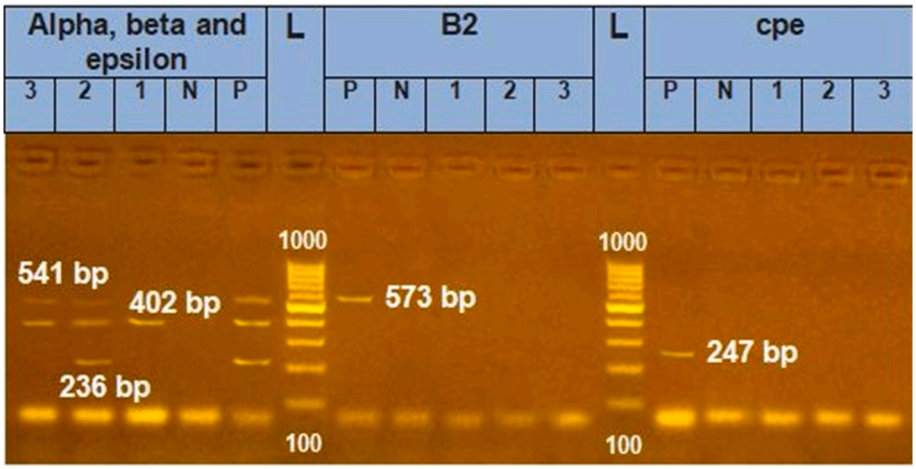

Fig. 1. A multiplex PCR for a represented *C. perfringens* isolates. The isolates showed the PCR product resulting from amplification of *plc* gene showing a fragment size of 402 bp, *cpb* gene showing a fragment size of 541 bp and *etx* gene showing a fragment size of 236 bp. All isolates showed negative results for *cpb2* and *cpe* genes.

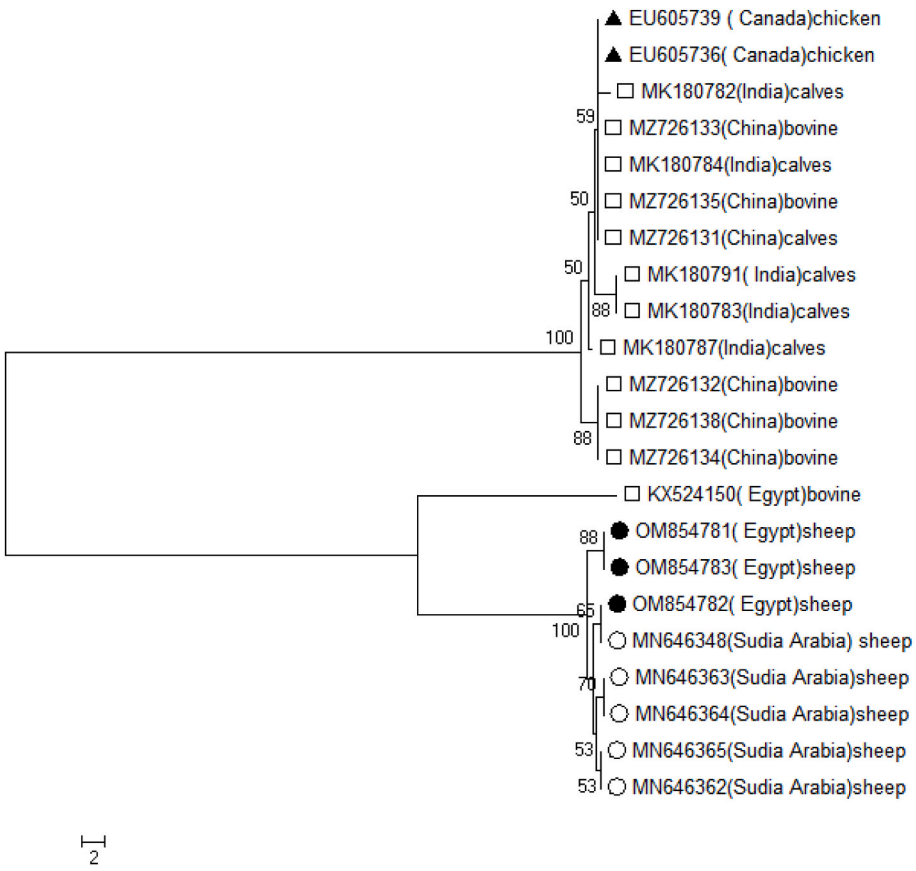

Fig. 2. phylogenetic tree for *plc* gene sequence of 3 Egyptian *C. perfringens* strains with other sequences from the GenBank.

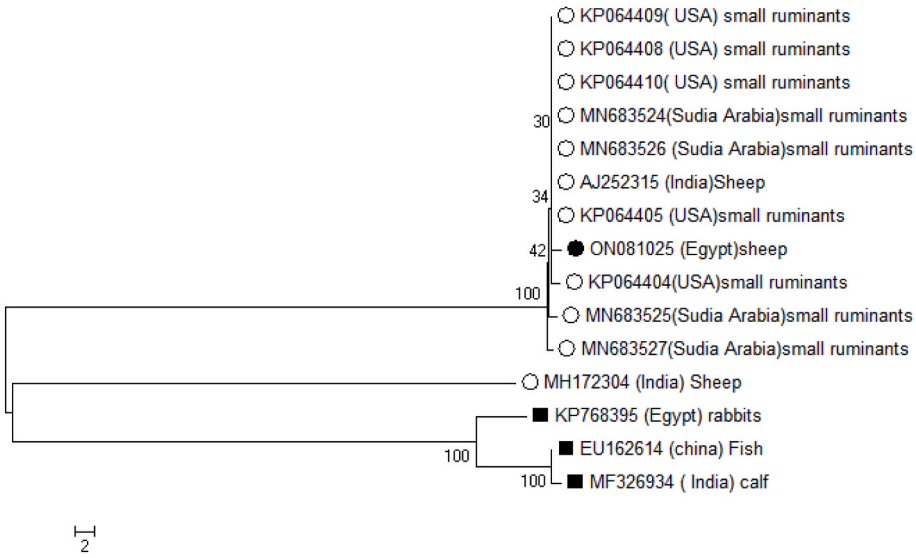

Fig. 3. Phylogenetic tree for *cpb* gene of *C. perfringens* strains with sequences from the Gene Bank.

The variation between different seasons may be due to climatic changes, management factors and hygienic conditions of housing [32–40].

Contrary to the findings of Omer et al. [41], we did not find enterotoxin gene (*cpe*) and *cbp2* gene in all detected isolates.

The gene coding to α-toxin in Egypt was found to show variability, and it is interesting that some of the strains are related to strains isolated from China. Such findings can be attributed to the fact that animal movement, animal importation and animals are transported between different countries, and hence, bacterial infection can easily be found in various places [42–44].

To carry out an extensive intra-specific diversity analysis that will help to enhance prevention and management of the spread of this bacterium, it is critical to understand the evolutionary links between *C. perfringens* isolates. According to phylogenetic analysis, the partial sequences of *C. perfringens* strains based on *plc* gene, *cpb* gene and *etx* gene obtained in the present study showed high degree of similarity with

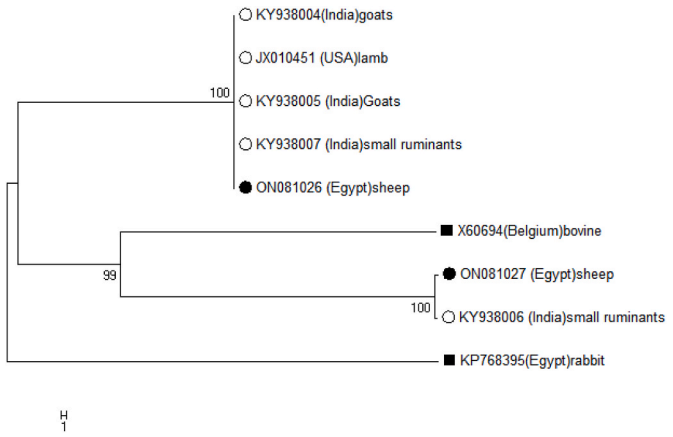

Fig. 4. Phylogenetic tree for *etx* gene of two Egyptian *C. perfringens* strains with sequences from the Gene Bank.

other sequences of *C. perfringens* strains isolating from sheep from Egypt and other countries. Indeed, the position of our Egyptian isolate on the phylogenetic tree, which was created, confirmed that the positive lambs had *C. perfringens* infection, as had previously been described by Nayel et al. [45] in the same country.

5. Conclusions

C. perfringens type A, B and D have been reported in lambs raising in Egypt. The multiplex PCR assay considered a useful and reliable tool for *C. perfringens* genotyping in routine veterinary diagnostics, and epidemiological studies of the prevalent types of *C. perfringens* in Egypt. The molecular survey is required for further investigation of *C. perfringens* strains in lambs and other species to apply an efficient control program.

Ethics approval and consent to participate

The study was performed according to guidelines and regulations of ethical committee of faculty of veterinary medicine, Benha University (BUFVTM22.09.2022). The study was conducting following ARRIVE guidelines.

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None.

Authors' contributions

Conceptualization, methodology, formal analysis, investigation, resources, data curation, writing-original draft preparation, S.M., A.S., I.Z., A.M. and R.A.; writing-review and editing, S.M., A.S., I.Z., A.M. and R.A.; project administration, S.M., A.S., I.Z. and A.M.; funding acquisition, S.M., A.S., I.Z. and R.A. All authors have read and agreed to the published version of the manuscript.

Consent for publication

Not applicable.

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

CRediT authorship contribution statement

Shymaa Moustafa: Writing – review & editing, Writing – original draft, Funding acquisition, Conceptualization. **Islam Zakaria:** Methodology, Funding acquisition, Formal analysis. **Abdelmoneim Moustafa:** Investigation, Formal analysis. **Rania AboSakaya:** Investigation, Funding acquisition. **Abdelfattah Selim:** Writing – review & editing, Writing – original draft, Methodology, Investigation, Funding acquisition, Conceptualization.

Declaration of Competing interest

There are no conflicts of interest declared by the authors.

Data availability

Data will be made available on request.

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